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July 31, 2015

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

1194

RE: GRAS Notification - Exemption Claim

Dear Dr. Gaynor,

Pursuant to the proposed 21C.F.R. § 170.36 (c) (l) Danisco US Inc. (operating as DuPont Industrial Biosciences) hereby claims that Alpha-amylase enzyme preparation produced by *Bacillus licheniformis* expressing the gene encoding alpha-amylase from *Geobacillus stearothermophilus* is Generally Recognized as Safe; therefore, it is exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(l)(i) The name and address of the notifier

Danisco US Inc.
925 Page Mill Road
Palo Alto, CA 94304

Proposed § 170.36 (c)(l)(ii) The common or usual name of notified substance

Alpha-amylase enzyme preparation from *Bacillus licheniformis* expressing the gene encoding the alpha-amylase from *Geobacillus stearothermophilus.*

Proposed § 170.36 (c)(l)(iii) Applicable conditions of use

The alpha-amylase is used in starch processing from grains, potatoes, cassava, and in brewing, cereal beverage manufacture and potable alcohol.

Proposed §170.36 (c)(l)(iv) Basis for GRAS determination

This GRAS determination is based upon scientific procedures.

Proposed § 170.36 (c)(l)(v) Availability of information

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A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

If you have questions or require additional information, please contact me at 650-846-5861 or fax at 650-845-6502.

Sincerely,

(b) (6)

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
Danisco US Inc.
(operating as DuPont Industrial Biosciences)
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Enclosures (3 binders)

#594

An α-amylase Enzyme

Preparation Derived from

Bacillus licheniformis

Expressing the α-amylase Gene

From

Geobacillus stearothermophilus

Is Generally Recognized As Safe

For Use in Food Processing

Notification Submitted by Danisco US Inc. (operating as DuPont Industrial Biosciences)

JULY 31, 2015

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1. GENERAL INTRODUCTION

The α-amylase enzyme preparation under consideration is produced by submerged fermentation of *Bacillus licheniformis* carrying the α-amylase gene from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) encoding the wild-type α-amylase enzyme.

The enzyme product is intended for use in grain and tuber starch processing, brewing, cereal beverage manufacture and potable alcohol. In these applications, the *G. stearothermophilus* α-amylase expressed in *B. licheniformis* will primarily be replacing α-amylase from one of the other available commercial sources. In these applications, α-amylase will either not be present in the final food or will be present in insignificant quantities as inactive residue, having no function or technical effect in the final food.

Other α-amylases currently in use include α-amylases from other microorganisms, most notably *Bacillus stearothermophilus, Bacillus licheniformis, Bacillus subtilis, Bacillus amyloliquefaciens,* and *Pseudomonas amyloderamosa.* Alpha-amylase expressed in *B. licheniformis* was determined to be GRAS by DuPont Industrial Biosciences in 2015.

The accepted name of the principle enzyme activity is glycogenase, endoamylase; 1,4-α-D-glucan glucanohydrolase.

The enzyme hydrolyzes of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units.

The EC number of the enzyme is 3.2.1.1 and the CAS number is 9000-90-2.

The information provided in the following sections is the basis of our determination of GRAS status of this α-amylase enzyme preparation.

As the α-amylase derived from *Geobacillus stearothermophilus* is was affirmed to be GRAS for use in food (21 CFR 184.1012), with no limitation other than cGMP, for the hydrolysis of edible starch to produce maltodextrins and nutritive carbohydrate sweeteners, the emphasis of this safety determination is to verify that the genetic modification and production process result in an enzyme preparation that is safe and suitable for its intended uses.

The safety evaluation in Section 7 focuses on the production strain, the manufacturing process, as well as a determination of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use (Pariza & Johnson, 2001; Pariza & Foster, 1983). The safety of the production organism (*B. licheniformis*) for the α-amylase is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; Pariza & Foster, 1983; IFBC, 1990; EU Scientific

Committee for Food, 1991; OECD, 1993; Berkowitz and Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36 (Appendix 1), DuPont Industrial Biosciences has determined that its α-amylase enzyme preparation produced by *Bacillus licheniformis* expressing the gene encoding α-amylase from *Geobacillus stearothermophilus* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

1.2 Name and Address of Notifier

Danisco US Inc.
(operating as DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

1.3 Common or Usual Name of Substance

The α-amylase enzyme preparation is from *Bacillus licheniformis* expressing the gene encoding the α-amylase from *Geobacillus stearothermophilus.*

1.4 Applicable Conditions of Use

The α-amylase is used as a processing aid in grain processing and tuber starch processing, brewing, cereal beverage manufacture and potable alcohol manufacture.

1.5 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.6 Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available for review and copying at 925 Page Mill Road, Palo Alto, CA 94304 or will be sent to the Food and Drug Administration upon request.

2. PRODUCTION ORGANISM

2.1 Production Strain

The production organism is a strain of *B. licheniformis* (strain BML612-Ethyl-4-CAP75), which has been genetically modified to over express a gene for the production of the *Geobacillus stearothemophilus* α-amylase, also referred to here as α-amylase. *Bacillus licheniformis* has been used for decades for the production of food enzymes with safety reviewed by De Boer et al (1994). The US Food and Drug Administration reviewed the safe use of food-processing enzymes from well-characterized recombinant microorganisms, including *B. licheniformis* (Olempska-Beer et al. 2006). An extensive environmental and human risk assessment of *B. licheniformis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. licheniformis* is not a human pathogen nor is it toxigenic. It is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001). The expression cassette was integrated into the host strain, at the *cat* locus, by Campbell-type recombination. The vector included the truncated *G. stearothermophilus amyS* gene encoding α-amylase under the regulation of the native *B. licheniformis amyL* promoter and terminator with the native *cat* gene. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated cat sequences.

2.2 Host Micoorganism

The host microorganism is *B. licheniformis* Bra7, which was developed from its wild-type parent, by classical strain improvement only, for optimal α-amylase production and lowered protease production. The parent strain *B. licheniformis* Bra7 and strains derived from it by Genencor (Formerly a division of Danisco now operating as DuPont Industrial Biosciences) have been in use for industrial scale production of α-amylase since 1989, with food grade versions in use for grain processing since 1998. *B. licheniformis* Bra7 has been used as host for production of multiple food enzymes previously notified to FDA as GRAS, including glycerophospholipid cholesterol acyltransferase (GRN 265) and maltotetraohydrolase (GRN 277).

A parent strain *Bacillus licheniformis* BML612 was developed from *Bacillus licheniformis* Bra 7 through deletion of sporulation capability, amylase activity, and chloramphenicol acetyltransferase activity.

2.3 Donor Microorganism

The donor strain used as a source for the α-amylase gene was *Geobacillus stearothermophilus* (formerly called *Bacillus stearothermophilus*) strain ASP-154. This strain was deposited in the American Type strain Culture Collection (ATCC) as *Bacillus stearothermophilus* ATCC 39709 by Enzyme Bio-Systems Ltd. (EBS), a subsidiary of CPC International Inc. The donor strain was

derived by classical microbiological methods from strain 55-9C6, isolated from soil by EBS in 1979. It is an asporogenic mutant ASP-154 obtained from 55-9C6.

2.4 Alpha-amylase Expression Cassette

The genetic modification of the *B. licheniformis* host involved recombinant DNA techniques to introduce multiple copies of the gene encoding the wild type truncated *G. stearothermophilus* α-amylase into the *B. licheniformis* host.

The modification employed a method by which a pUB110 and pE194 derived plasmid, containing the truncated *G. stearothermophilus amyS* gene encoding α-amylase under the regulation of the native *B. licheniformis amyL* promoter and terminator with the native *cat* gene, is introduced into the host strain. After integration all vector sequences of the plasmid were deleted which resulting a strain which only the *G. stearothermophilus* (formerly called *B. stearothermophilus*) *amyS* gene and the native *cat* gene were introduced into the host strain.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm the copy number of the integrated α-amylase cassette and the absence of bacterial vector DNA.

2.5 Stability of the Introduced Genetic Sequences

The production strain is completely stable after industrial scale fermentation as judged by α-amylase production using the production organism containing the integrated expression cassette.

2.6 Antibiotic Resistance Gene

No new antibiotic resistance genes were introduced in the construction of the production microorganism.

2.7 Absence of the Production Organism in the Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1 CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by IFBC (1990) is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

IUB Nomenclature	4-α-D-glucan glucanohydrolase
IUB Number:	3.2.1.1
CAS Number:	9000-90-2
Reaction catalyzed:	Endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units
Other names:	glycogenase, endoamylase; 1,4-α-D-glucan glucanohydrolase

3.2 Amino Acid Sequence

The amino acid sequence of *G. stearothermophilus* α-amylase enzyme is shown in Appendix 2. The *AmyS* gene from the strain *G. stearothermophilus* ASP-154 encodes an α-amylase enzyme, the truncated sequence of which encoding the mature protein also referred to as Ethyl 4 α-amylase is listed in Appendix 2. The sequence of the α-amylase is identical to the *G. stearothermophilus* α-amylase enzyme that is affirmed as GRAS according to 21CFR184.1012.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the α-amylase enzyme which follows standard industry practice (Kroschwits, (1994); Aunstrup *et al.*, 1979; Aunstrup 1979). For a diagram of the manufacturing process, see Appendix 3. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this α-amylase concentrate are standard ingredients used in the enzyme industry (Kroschwits, 1994; Aunstrup *et al.*, 1979; Aunstrup, 1979). All the raw materials conform to the specifications of the Food Chemicals Codex (FCC), 9th edition (US Pharmacopeia, 2014), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been set in line with FCC and JECFA requirements and acceptability of use for food enzyme production. DuPont industrial Biosciences uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam used in the fermentation and recovery is used in accordance with cGMP per the FDA correspondence to ETA acknowledging the listed antifoam dated September 11, 2003. The maximum use level of the antifoam in the production process is $\leq$0.15%.

Glucose (which may be derived from wheat) and soy flour will be used in the fermentation process and will be consumed by the microorganism as nutrients. No other major allergen substances will be used in the fermentation, or in recovery processes and the formulation.

4.2 Fermentation Process

The α-amylase enzyme is manufactured by submerged fermentation of a pure culture of the genetically modified strain of *B. licheniformis* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed, and main fermentor) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed necessary. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process.

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation or filtration;
2. Concentration – ultrafiltration;

3. Addition of stabilizers/preservatives;
4. Polish filtration.

4.4 Formulation/standardization

The ultrafiltered concentrate is stabilized by final formulation to contain up to 17% sorbitol, 11% Sodium chloride, 1.0% Propylene glycol, 1.0% Potassium sorbate, 0.1% Calcium chloride and 0.1% Paraben (methyl-, propyl-) at pH 5-6.7. The remaining is water.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The liquid concentrate is stabilized with the formulation ingredients listed below and tested to demonstrate that it meets the specifications.

Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition:

Enzyme activity	13400-14600 AUU/g
Sorbitol	14.0-17.0%
Sodium chloride	8.0-11.0%
Calcium chloride	0-0.1%
Propylene glycol	0-1.0%
Paraben (methyl-, propyl-)	0-0.1%
Potassium sorbate	0-1.0%
Remaining is water	
pH	5-6.7

5.2 Specifications

Alpha-amylase meets the purity specifications for enzyme preparations set forth in the Food Chemical Codex 9th edition (US Pharmacopeia, 2014). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint Evaluation Committee of Food Additives (JECFA) in the Compendium of Food Additive Specification (JECFA, 2006).

The results of analytical testing of the 3 lots of product is given in Appendix 4 verifying that meets FCC 9th edition (2014) and JECFA (2006) specifications for enzyme preparations.

6. APPLICATION

6.1 Mode of Action

The α-amylase endohydrolyzes (1→4)-α-D-glucosidic linkage in polysaccharides containing three or more (1→4)-α-linked D-glucose units. It acts on starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the α-configuration (the initial anomeric configuration of the free sugar group released).

6.2 Uses and Use Level

Ethyl 4 α-amylase is used as a processing aid in starch, brewing, cereal beverage manufacture, potable alcohol and fuel ethanol with resulting distillers' grains used as animal feed.

6.2.1 Uses

The enzyme product will be used in the following applications:

Starch processing

The α-amylase will be used in combination with other enzymes for the manufacture of glucose from granular starch from various sources including corn, wheat, milo, barley, rice, potatoes and cassava. The resultant glucose-rich syrups can be purified to meet various specifications: crystallized to produce dextrose, isomerized to produce high fructose corn syrup, or may be fermented to produce organic acids, alcohol or amino acids (potable alcohol as a fermentation based end-product is discussed below, organic acids and amino acids may be incorporated at a later date). The purification process for glucose and fructose syrups production will include carbon ion exchange (large local pH swings) and evaporation at temperatures up to 85 ^{o} C for 30 minutes or less.

The α-amylase may also be used to treat liquefied starch for the manufacture of starch syrups with special saccharide distribution. The process will involve evaporation of the syrups, at temperatures up to 85 ^{o}C for 30 minutes or less.

Brewing and Cereal Beverage

The α-amylase is used to maximize the conversion of starchy substrate to fermentable carbohydrate. It will be used in the extraction and saccharification of starch (mashing) from malted cereal, cereal and other plant sources (includes barley, corn, wheat, rye, milo, rice,

tapioca and potatoes). The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol (and sometimes organic acids). The α-amylase may also be used in the fermentation vessel hydrolyzing liquified starch to glucose which is fermented to ethanol.

Potable Alcohol

The α-amylase will be used in combinations with other enzymes (glucoamylase, proteases, etc.) to maximize the conversion of starchy substrate to fermentable carbohydrate. After saccharification and fermentation are completed, the slurry goes through distillation at ~85° C. The water phase goes to evaporation and the solids go to dryers. Denatured enzyme ends up in the Distillers' Grains. In all of these applications, the enzyme product will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no function in the final food.

6.2.2 Use Levels

The α-amylase enzyme preparation is used at the minimum level required to achieve the desired effect and in accordance with the principles of current Good Manufacturing Practice (GMP).

The α-amylase will be used in grain processing in the manufacture of high fructose corn syrup (HFCS), which will then be used in soft drinks. The proposed application rate of α-amylase is 0.88-8.8 TOS/kg dry starch.

In a typical mash brewing and cereal beverage application, the recommended dosage for α-amylase is 3.2-31.6 mg TOS/kg grist.

During the distillation process of potable alcohol, the proposed application rate is 2.9-29.1 mg TOS/kg dry substances.

6.3 Enzyme Residues in the Final Foods

As noted above, the α-amylase is expected to be inactivated or removed during the subsequent production processes for all applications. The enzyme is added during carbohydrate processing after the liquefaction step. After that, the glucose rich syrup or starch syrup obtained goes through several purification steps (filtration, carbon treatment, ion exchange, etc.), so no carryover of the α-amylase is expected.

In brewing and cereal beverage, the enzyme product is added in mashing, After mashing, the wort is separated from the spent grains via filtration and ultimately boiled for 1-1.5 hrs. for sterilization. With a temperature of 100 °C during this process the enzyme product will be completely inactivated.

In potable alcohol production, the alcohol is distilled after the α-amylase is used, so the alcohol does not contain the α-amylase.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC, 1990). Pariza and Foster (1983) define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *Bacillus licheniformis* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

7.1.1 Safety of the host

B. licheniformis is a known safe host for enzyme production and is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. The safety of *B. licheniformis* strains was reviewed by De Boer et al (1994). *B. licheniformis* is considered to be a benign organism that does not possess traits that cause disease. This also applies to the DuPont Industrial Biosciences *B. licheniformis* host strain, which has been demonstrated to be non-pathogenic, non-toxigenic and not cytotoxic.

The potential risk associated with the use of this bacterium in fermentation facilities is low (US EPA, 1997).

Recently scientists with the US Food and Drug Administration reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. licheniformis* (Olempska-Beer *et al.,* 2006). An extensive risk assessment of *B. licheniformis*, including its history of commercial use has been published by the US EPA (1997). It was concluded that *B. licheniformis* strains used for enzyme manufacture are neither pathogenic nor toxigenic to humans.

Mixed carbohydrase and protease preparation from *B. licheniformis* was affirmed as Generally Recognized as Safe (GRAS) for use as direct food ingredients in the US Code of Federal Register (21CFR184.1027). In addition, (GRAS) Notices have been submitted to the US FDA for several food enzymes from genetically modified *Bacillus licheniformis* strains, including pullulanase (GRN 72), α-amylase (GRN 22, GRN 24 and GRN 79), glycerophospholipid

cholesterol acyltransferase, GCAT (GRN 265), maltotetraohydrolase (GRN 277) and xylanase (GRN 472). Based on the information provided in these GRAS Notices, the agency did not question the conclusion that food enzyme preparations from *B. licheniformis* are GRAS under the intended conditions of use.

In various countries enzyme preparations derived from *B. licheniformis* have been formally approved, e.g. Canada (α-amylase, protease, pullulanase and xylanase)see Canadian List of Permitted Food Enzymes (Lists of Permitted Food Additives, http://www.hc-sc.gc.ca/fn-an/securit/addit/list/5-enzymes-eng.php), France (α-amylase, protease, pullulanase and cyclomalto-dextrine glucotransferase, see Arrêté du 19 Octobre 2006 and xylanase, see Arrêté du 30 janvier 2015), and Australia/New Zealand (α-amylase, pullulanase, see Australian Standard 1.3.3).

Also JECFA completed favorable evaluations for α-amylase and pullulanase produced by *B. licheniformis* (JECFA 1987, 2004).

The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. In 2007, the Scientific Committee set out the overall approach to be followed and established the first list of the biological agents. The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ). If a defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. The safety of *B. licheniformis* as a production organism has been assessed by EFSA and been accorded QPS status provided the qualification requirements are met (see http://www.efsa.europa.eu/en/topics/topic/qps.htm?wtrl=01). For *Bacillus* strains the specific requirement is absence of toxigenic activity, which has been tested for the host strain.

The species *B. licheniformis* is accepted as a safe host for the construction of Risk Group I GMMs in several countries, like Germany, The Netherlands, and etc. and is exempted as a host under the NIH Guidelines in the USA. It is also on the Tier1 exempt list used by the US EPA, exempting the species from standard notification requirements under the TSCA Biotechnology Rule.

Despite the documented safety of *Bacillus licheniformis,* several strains derived from the *B. licheniformis* safe strain lineage and comparable to the current production strain were tested for pathogenicity and toxicity by DuPont Industrial Biosciences (see Appendix 5). The conclusion of the research was that no toxic substances were produced by the strain, i.e. that it is non-pathogenic and non-toxigenic.

The production organism of the α-amylase enzyme preparation, the subject of this submission is a strain of *B. licheniformis*, BML612-Ethyl-4-CAP75, which has been genetically modified to over express a gene for the production of the *G. stearothermophilus* α-amylase.

The host strain is *B. licheniformis* Bra7, which was developed from its wild-type parent, by classical strain improvement only, for optimal α-amylase production and lowered protease production. The

parent strain *B. licheniformis* Bra7 and strains derived from it have been in use for industrial scale production of α-amylase since 1989, with food grade versions in use for grain processing since 1998.

From the information reviewed, it is concluded that the production organism *B. licheniformis* strain BML612-Ethyl-4-CAP75 provides no specific risks to human health and is safe to use as the production organism of α-amylase. The strain is non-pathogenic and non-toxigenic.

7.1.2 Safety of the Donor Organism

The donor strain used as a source for the α-amylase gene was *Geobacillus stearothermophilus* (formerly called *Bacillus stearothermophilus*) strain ASP-154. This strain was deposited in the American Type strain Culture Collection (ATCC) as *Bacillus stearothermophilus* ATCC 39709 by Enzyme Bio-Systems Ltd. (EBS), a subsidiary of CPC International Inc.

The donor strain was derived by classical microbiological methods from strain 55-9C6, isolated from soil by EBS in 1979. It is an asporogenic mutant ASP-154 obtained from 55-9C6.

G. stearothermophilus is already used as a source for producing commercial food enzymes. Alpha-amylase from *G.stearothermophilus* was evaluated during the 37th meeting of the Joint FAO/WHO Expert Committee on Food Additives in 1990, an ADI "not specified" was established (JECFA, 1990). Alpha-amylase from *G. stearothermophilus* was also affirmed as GRAS by FDA (21CFR184.1012).

Apart from the U.S. *G. stearothermophilus* α-amylase gene, no genetic material other than from the host itself is introduced in the modified strains, and the non-toxic and non-pathogenic status of *B. licheniformis* is well established.

Alpha-amylase from *B. stearothermophilus* (nowadays called *G. stearothermophilus*) has been affirmed as GRAS by the FDA (FDA, 1994). Numerous feeding, mutagenicity, and carcinogenicity studies using enzyme product from these strains have been performed, and no evidence of a toxic or mutagenic effect has been observed. Reports of subchronic toxicity studies performed using the α-amylase from its natural and recombinant sources have been published (MacKenzie *et al*, 1989).

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of α-amylase is conducted in a manner similar to other food and feed enzyme production processes. It consists of a pure-culture fermentation process, cell separation, concentration and formulation. The process, described in Appendix 3 is conducted in accordance with food Good Manufacturing Practice (GMP) as set forth in 21 CFR Part 110. The resultant product meets the general requirements for enzyme preparations of the FCC, 9th edition (US Pharmacopeia, 2014) and JECFA (2006) enzyme specifications.

Although glucose possibly from wheat as well as soy flour are used in the fermentation process, as they will be consumed by the microorganism as nutrients, and will not pose any allergy risk in the final product.

7.3 Safety of *Bacillus licheniformis* α-amylase

7.3.1 Allergenicity

According to Pariza and Foster (1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. α-amylase has been used in food processes for many years and has generated no known safety concerns.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residue in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy (Bindslev-Jensen *et al.*, 2006).

Despite this lack of general concern, the potential that Ethyl 4 α-amylase could be a food allergen was assessed by comparison with sequences of known allergens. Based on the sequence homology alone, it was concluded that the *B. licheniformis* α-amylase is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Alimentarius Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The *G.stearothermophilus* Ethyl 4 α-amylase mature protein sequence expressed in *B. licheniformis* is given below in FASTA format.

AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGTSRSDVGYGVYDLYDLGEF
NQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQI
QAWTKFDFPGRGNTYSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLMYAD
LDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSYVRSQTGKPLFTVGEYWSYDINK
LHNYITKTNGTMSLFDAPLHNKFYTASKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSW
VDPWFKPLAYAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQHDYLDHSDIIGWTR
EGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKVFYDLTGNRS

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database (http://www.allergenonline.org/index.shtml) containing 1897 peer-reviewed allergen sequences (listed in http://www.allergenonline.org/databasebrowse.shtml) revealed multiple stretches throughout the peptide sequence with over 35% identity to TAKA-amylase-A. TAKA-amylase-A is an α-amylase, EC 3.2.1.1, from *A. oryzae* (NCBI gi|94706935|sp|POC1B3.1|AMYA1_ASPOR), which is also referred to as Asp o 21, an environmental allergen. The maximum sequence identity to the allergen was 38%.

FASTA alignment of the above sequence with known allergens using the AllergenOnline database (http://allergenonline.org/index.shtml) confirmed the matches (using E-value <0.1 as the cut-off) with TAKA-amylase-A or Asp o 21.

Although alpha-amylase from *A. oryzae* is an occupational allergen (Skamstrup Hansen *et al.*, 1999), allergy symptoms after ingestion of the enzyme have been reported only for four individuals, either by consumption of bread baked with the enzyme (Baur & Czuppon, 1995; Kanny & Moneret-Vautrin, 1995; Moreno-Ancillo *et al.*, 2004) or after oral challenge with α-amylase (Losada *et al.*, 1992). Other studies with patients with documented occupational or other allergies revealed no cases of food allergy to α-amylase from *A. oryzae* or other commercial enzymes used in food (Skamstrup Hansen *et al.*, 1999; Bindslev-Jensen *et al.*, 2006). Thus, food allergy to α-amylase from *A. oryzae* is extremely rare. TAKA-amylase A is not identified as food allergens (Allergen Nomenclature, International Union of Immunological Societies (IUIS; www.allergen.org).

Although cautioned against in Codex (2009), researched by Herman *et al.* (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org that there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%), this database does allow for

isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing this search produced no sequence matches with known allergens.

Microbial enzymes acting as environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a recently published study (Bindslev-Jensen *et al.*, 2006) that failed to indicate positive reactions to 19 orally challenged commercial enzymes in a double blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a concern with regard to food allergy.

In conclusion, based on the sequence homology alone, *Geobacillus stearothermophilus* α-amylase expressed in *Bacillus licheniformis* is unlikely to pose a risk of food allergenicity. *G. stearothermophilus* α-amylase has been safely used in food processing for decades, without any reports of food allergenicity.

As for all enzyme products, an MSDS for the α-amylase product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

7.3.2 Safety of use in food

In addition to the allergenicity assessment described above, the safety of this α-amylase has also been established using the Pariza and Johnson (2001) decision tree:

1. **Is the production strain[1] genetically modified[2,3]?**
   Yes → go to 2.

2. **Is the production strain modified using rDNA techniques?**
   Yes → go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA[4,5] have a history of safe use in food[6]?**

   Yes, α-amylase has been used for years in food processing. The *G. stearothermophillus* α-amylase is not new in food processing. It is homologous to the *G. stearothermophillus* α-amylase affirmed as GRAS by FDA (21CFR 184.1012), and its protein sequence is not similar to known sequences of food allergens. In addition, the enzyme will be inactivated in the food manufacture process. → go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[7]?

Yes, no transferable antibiotic resistance gene DNA is present in the enzyme preparation → go to 3e.

3e. Is all other introduced DNA well characterised and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

Yes, inserted DNA is well characterized and free of unsafe attributes. Go to 4.

4. Is the introduced DNA randomly integrated into the chromosome? No, it is introduced in the *cat* locus. *Go to 6.*

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[8]? Yes. The *B. licheniformis* Bra7 safe lineage is well-established as presented in Appendix 5. Its safety as a production host and methods of modification are well-documented, and the safety of the resulting enzyme preparations have been confirmed through repeated toxicology testing (see Appendix 5).

Conclusion: Article is accepted.

Based on the publicly available scientific data from the literature and additional supporting data generated by DuPont, company experts trained in the field of enzyme safety evaluation have concluded that *Geobacillus stearothermophilus* α-amylase expressed in *Bacillus licheniformis* strain BML612-Ethyl-4-CAP75 is safe and suitable for use in the grain and tuber starch processing, as well as brewing, alcoholic beverages, and potable alcohol. Further, the α-amylase is Generally Recognized as Safe (GRAS) for those uses, which was concurred to by Dr. Michael Pariza based on his expert review (Appendix 6).

[1] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this

[2] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (e.g., UV or chemically-induced mutagenesis) or rDNA technologies.

[3] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

[4] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[5] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[6] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

[7] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm)

Although the Pariza and Johnson evaluation resulted in the conclusion to accept the enzyme preparation as safe without new toxicology testing, the safety of the enzyme preparation in the intended use was further confirmed using existing toxicological information through the scientific procedure as described below.

7.3.3 Safety Assessment

Toxicology studies with α-amylase from *B. licheniformis* production strain have not been conducted. However, in addition to the general recognition that the expressed *G. stearothermophilus* α-amylase protein has a history of safe use, DuPont has determined by scientific procedures that its production organism *B. licheniformis* pertains to a safe strain lineage. A review of numerous toxicology studies conducted with enzyme preparations produced by different DuPont strains of *B. licheniformis* indicates that, regardless of the production organism strain, all enzyme preparations are not irritating to the skin and eyes, are not skin sensitizers, are not mutagenic or clastogenic in genotoxicity assays and do not adversely affect any specific target organ (Appendix 5). Due to the consistency of the findings from enzyme preparations derived from different *B. licheniformis* strains all pertaining to the same lineage, it is expected that any new enzyme preparation produced from *B. licheniformis* strains would behave similarly from a toxicological standpoint.

Using the concept of safe strain lineage (Pariza and Johnson, 2001) endorsed by the Enzyme Technical Association and accepted by regulatory agencies, the enzyme and production strain most closely related to α-amylase is EBS2 amylase from *B. licheniformis* strain. EBS2 amylase is a modified amylase and toxicology data has been generated for this enzyme. Data from EBS 2 α-amylase can be extrapolated to α-amylase and this approach is in line with the safe strain lineage concept.

In this assessment, toxicology data obtained EBS2 α-amylase from *B. licheniformis* are applied to α-amylase from *Bacillus licheniformis* since both production organisms are derived from the same BML 612 lineage and both have inserted *G. stearothermophilus* α-amylase gene. Extrapolation of toxicology information is in line with the safe strain lineage concept of Pariza and Johnson (2001).

DuPont Industrial Biosciences has conducted five studies on EBS2 α-amylase enzyme produced from *B. licheniformis*. The results are evaluated, interpreted and assessed in this document. The test material, an ultra-filtrate concentrate (UFC) used in all toxicology investigations, has the following characteristics:

Description:	Clear brown liquid
Total protein:	16.36 mg total protein/ml
TCA protein:	5.52 mg/ml.
TOS:	17.92 %
Enzyme activity:	7894 AAU/ml

The studies include:

A. Acute dermal irritation study in rabbits

B. Acute eye irritation/ corrosion study in rabbits

C. Bacterial reverse mutation assay – Ames assay

D. In vitro mammalian chromosomal aberration test performed with human lymphocytes

E. 13-week oral (gavage) toxicity study in CD rats

All safety studies were conducted in accordance with internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practices ("GLP") according to the FDA/OECD.

Summaries are included below.

A. Acute dermal irritation study in rabbits (sequential approach) (Scantox Study, 2005a)

1) Procedure:
The objective of this study is to assess the acute dermal effect of Alpha Amylase (EBS2). This study was investigated according to the method recommended in the OECD Guideline No. 404 and EEC Directive Annex I, II, III and IV, Official Journal of the European Communities published in 1993.

In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and reading was made at 1, 24, 48 and 72 hours post application.

2) Results
No deaths and overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays.

3) Evaluation
The mean score for skin irritation was 0. Under the conditions of this assay, Alpha amylase (EBS-2) is not a skin irritant.

B. Ocular Irritation in the Rabbit (Scantox study, 2005b)

1) Procedure

The objective of this study is to assess the ocular irritation potential of Alpha Amylase (EBS2). This study was investigated according to the method recommended in the OECD Guideline No. 405 and EEC Directive, part B5, Official Journal of the European Communities published in 1992.

In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded 1 and 24 hours later. The right eye served as control. After the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage at 48 and 72 hours after the treatment. A confirmatory test was conducted with 2 rabbits.

2) Results

In the initial study, slight conjunctivitis was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay, slight conjunctival irritation was observed at the 1-hour observation period with clearing by 24 hours.

3) Evaluation

The primary eye irritation score was 0.3. Under the conditions of this assay, Alpha Amylase (EBS2) is not an eye irritant.

C. Bacterial Reverse Mutation Assay – Ames assay (Scantox Study, 2005c)

1) Procedure:

The objective of this assay is to assess the potential of Alpha Amylase (EBS2) to induce point mutation (frame-shift and base-pair) in five bacterial tester strains: *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver S-9). The doses selected for the two main assays were based on results from a preliminary toxicity test. The positive controls used for assays without S-9 mix were sodium azide, 2-nitrofluorene, 9-amino acridine and cumene hydroperoxide and the positive control used for assays with S-9 mix was 2-aminoanthracene. The tests were performed using the "treat and plate" method to avoid the possibility of interference from histidine in the test article. In the treat and plate method, various concentrations of Alpha Amylase (EBS2) were mixed with a concentrated bacterial suspension and nutrient broth. After a period of incubation, bacteria were separated by sedimentation, re-suspended with buffer and mixed with top agar. The whole process was repeated twice and then the mixture was spread on selective agar plates. The plates were incubated at 37°C for 72 hours and the number of revertant colonies on each plate was counted. Triplicate plates were used for each dose level and the whole assay was repeated twice. This assay was conducted in accordance with OECD guideline No. 471 (Bacterial Reverse Mutation Test).

2) Results:
Based on the results of the preliminary assay, the following dose levels were selected for the first main assay: 0.5, 1.6, 5, 16 and 50 ug total protein/plate for TA 98, TA 100, TA 102 and TA 1535 in the absence of S-9 mix and 1.6, 5.0, 16, 50 and 160 µg total protein/plate for TA 98, TA 100, TA 102 and TA 1535 in the presence of S-9 mix. In the second main test, dose levels ranging from 1.6 to 5000 µg/plate were selected TA 100, TA 102 and TA 1535 both in the presence and absence of S-9 mix and doses ranging from 0.16 to 160 µg/plate were used for TA 98 and TA 1537 in both presence and absence of S-9 mix. The highest dose used, 5000 ug total proteins/plate, is the maximum dose level required by OECD guideline No. 471.

No biologically or statistically significant increases in the number of revertant colonies were observed in any tester strain after treatment with Alpha Amylase (EBS2) at any dose level, either in the absence or presence of S-9 mix.

Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

3) Evaluation
Under the conditions of this assay, there is no evidence to suggest that Alpha Amylase (EBS2) is a mutagen in the Ames assay.

D. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes (Scantox Study, 2005d)

1) Procedure
The objective of this assay is to investigate the potential of Alpha Amylase (EBS2) to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). Alpha Amylase (EBS-2) was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S-9).

A preliminary toxicity test and two main tests were performed. In the preliminary toxicity test and the first main test, all cultures were treated for 3 hours. In the second main test, cultures without S-9 mix were treated for 20 hours and those with S-9 mix for 3 hours.

Cultures with S-9 mix were incubated at 37°C with gentle mixing for 3 hours, and then centrifuged and the supernatant removed and discarded. The cells were resuspended in fresh medium for a further 17 hours until harvest. Two hours prior to the scheduled cell harvest time, Demecolcine (0.1 µg/ml) was added to all cultures.

Cultures without S-9 mix were incubated at 37°C were gentle mixing for 20 hours, then centrifuged and the supernatant discarded. Two hours prior to the scheduled 20-hour harvest time, Demecolcine (0.1 µg/ml) was added to the cultures.

At the 20-hour harvest time, metaphase cells were harvested by centrifugation and re-suspended in appropriate medium. The cells were then fixed on slides and stained. For each culture, the number of cells at metaphase were counted in 1000 cells. The mitotic index was calculated as the percentage of cells at metaphase.

Slides from cultures treated with 3 concentrations of Alpha Amylase (EBS2) were analyzed for metaphase. Daunomycin was used as the positive control in the non-activated assay and cyclophosphamide was the positive control in the activated assay.
This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test).

2) Results
In the preliminary dose-range assay, severe cytotoxicity was observed at 1600 and 5000 µg/ml in both non-activated and activated cultures. Moderate cytotoxicity was observed in cultured treated with 500 µg/ml. Based on these results, the highest test concentration that could be selected was 800 µg/ml.

In the first main test, doses of 200, 400 and 600 µg/ml were used for both activated and non-activated cultures. In the second main test, doses of 50, 100 and 200 µg/ml were used for cultures without S-9 mix and doses of 200, 400 and 600 µg/ml were selected for cultures with S-9 mix.

A reduction in mitotic index was noted at the highest dose level in both main tests (52 and 61% reduction, respectively) and this level of toxicity meets the requirements of OECD 473 guideline for the highest concentration to be scored for aberrations (> 50% reduction in mitotic index).

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with Alpha Amylase (EBS-2) both in the presence and absence of metabolic activation (S-9 mix). Significant increases in aberrant metaphases were demonstrated with the positive controls.

3) Evaluation
Under the conditions of this test, there is no evidence to suggest that Alpha Amylase (EBS2) induces chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

E. A 13-week Oral (Gavage) Toxicity Study in Rats (ScantoxStudy, 2005e)

1) Procedure
The objective of this study was to investigate the potential of Alpha Amylase (EBS2) to induce systemic toxicity after repeated daily oral administration. Groups of 10 Sprague Dawley rats/sex each were gavaged daily with 0 (water for injection in 5.9% NaCl), 16, 32, or 64 mg total protein/kg body weight in a constant volume of 10 ml/kg body weight. These doses

corresponded to, respectively, 0, 175, 350 or 700 mg TOS/kg bw/day. The sodium content in the test material was 15%. Since the amount of NaCl given to the high dose was 585 mg/kg bw/day in 10 ml dosing volume (1 ml = 58.5 mg/ml), the control group was dosed with the same amount of NaCl.

All animals were observed daily for mortality and signs of morbidity. Animals of the same sex were pair-housed in transparent polycarbonate cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. All groups were housed under controlled temperature, humidity and lightning conditions.

Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. During week 12, a functional observation battery and behavior observation were performed on all animals. Hematology and clinical chemistry were conducted at study termination prior to necropsy, which was performed on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination of selected organs was conducted first on control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408.

2) Results

There were no treatment-related deaths in this study. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, hematology, clinical chemistry, absolute and relative organ weights, clinical observations, and ophthalmologic examinations. There were no treatment-related histopathologic changes. In the functional observation battery testing, there were no statistically significant changes noted in treated groups.

3) Evaluation

Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established as the highest dose tested (64 mg total protein/kg bw/day or 700 mg TOS/kg bw/day).
In toxicology studies conducted with EBS2 alpha amylase from *B. licheniformis* , it is not an eye or skin irritant. EBS2 α-amylase was not mutagenic in the bacterial reverse mutation assay (Ames assay) and was not clastogenic in the mammalian system (*in vitro* chromosomal aberration assay with human peripheral lymphocytes) in both the presence and absence of metabolic activation. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established as the highest dose tested (64 mg total protein/kg bw/day or 700 mg TOS/kg bw/day).

7.4 Overall Safety Assessment and Human Exposure

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, a NOAEL was established at 64 mg TP/kg bw/day (equivalent to 700 mgTOS/kg bw/day). The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to α-amylase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 64 mg TP/kg bw/day
= 700 mg TOS/kg bw/day

7.4.2 Human Exposure to Ethyl 4 α-amylase

1) Uses and Applications – Liquid Foods

a. Brewing

In a typical mash brewing application, the recommended dosage for this α-amylase is 31.6 mg TOS/kg grist. Approximately 17 kg of grist (e.g. malted barley) are used to make 1 hectolitre (100 litres) of finished beer. Hence,

31.6 mg TOS/kg grist x 17 kg/100 liters beer =
537.2 mg TOS/100 liters beer =
5.37 mg TOS/liter beer

b. Potable Alcohol

The maximum application rate of this α-amylase in ethanol for the production of potable alcohol is 29.1 mg TOS/kg RM (grist). The estimated yield of alcohol is 35% Therefore,

29.1 mg TOS/kg grist =
29.1 mg TOS/0.35 L potable alcohol =
83.1 mg TOS/L potable alcohol

c. Cereal Beverage

In the absence of consumption data on cereal beverage, the consumption of whole milk is used to represent a worst case scenario. The proposed application rate of α-amylase in cereal drink is 31.6 mg TOS/kg dry cereal. Since 20 L cereal beverage can be made from 1 kg dry cereal, the concentration of α-amylase in rice beverage is:

31.6 mg TOS/kg dry cereal = 31.6 mg TOS/20 liters cereal beverage =
1.58 mg TOS/liter of cereal beverage

d. Starch and carbohydrate processing

This α-amylase is used in grain processing in the manufacture of high fructose corn syrup (HFCS) which will then be used in soft drinks. The proposed application rate of α-amylase is 8.8 mg TOS/kg dry starch (worst case).

Food products from starch processing fall in the category of both liquid and solid foods. Typically foods derived from starch processing are syrups (e.g. High Fructose Corn Syrup, HFCS), sweeteners and modified starch.

1) Ratio between grain and starch 0.55 kg starch/kg grain

Starch processes start with starch originating from grist as the raw material.

2) Ratio between starch and syrup 1 kg starch/kg syrup

Typically 1 kg of sweetener is produced per 1 kg starch.

3) Ratio between syrup and final beverage 0.12 kg HFCS/L soft drink

Syrups and sweeteners are mostly applied in soft drink beverages and are therefore considered to be part of the category of liquid foods. Soft drinks typically contain 10-14% w/v HFCS so on average 120 g HFCS per L. The typical ratio is 0.12 kg starch/L final beverage.

8.8 mg TOS/kg starch x 0.12 kg starch/ L final beverage =
105.6 mg TOS/100 liters beverage =
1.06 mg TOS/kg final beverage

However, for the purpose of selecting an overall maximum exposure via liquids, the worst case TOS concentration in beer (5.37 mg TOS/L) is appropriate, because:

- The application rate of the α-amylase to grist used in brewing and cereal beverage is higher than that to grist for potable alcohol (distilled spirits) and HFCS sweetened soft drinks.
- Taking the respective process yields into account, the resulting worst-case exposure in beer is higher (on an equal alcohol content basis) than in either cereal beverages or sweetened soft drinks or the theoretical exposure via potable alcohol. It is reasonable to equalize intake based on % alcohol, as the maximum intake of any alcoholic drink will be limited largely by the maximum intake of alcohol the body can tolerate, not by the volume of the drink. Thus, while it is possible that 25% of the daily liquid intake of an adult individual were to consist of beer (i.e.1.5L for a 60-kg adult), it is inconceivable that could be the case for distilled spirits.
- Moreover, in distilled spirits the actual TOS concentration will be minimal compared to the maximum theoretical TOS concentration, as the enzyme protein and other organic solids will be removed in the distillation step.

Hence, the higher exposure from brewing was used in our risk assessment to represent a worst case scenario exposure via intake of liquids regardless of whether this is from consumption from beer, cereal beverage, or distilled spirits, with the assumption that 25% of all consumed beverages are manufactured from grist treated with the α-amylase.

2) Uses and Applications – Solid Foods

a. Starch and carbohydrate processing

This α-amylase is used in grain processing in the manufacture of high fructose corn syrup (HFCS), which will then be used in bread and diary. The proposed application rate of α-amylase is 8.8 mg TOS/kg starch.

Food products from starch processing fall in the category of both liquid and solid foods. Typically foods derived from starch processing are syrups (e.g. High Fructose Corn Syrup, HFCS), sweeteners and modified starch.

1) Ratio between grain and starch 0.55 kg starch/kg grain

   Starch processes start with starch originating from grist as the raw material.

2) Ratio between starch and modified starch or sweeteners 1 kg starch/kg mod. starch

   (Modified) starches or sweeteners are applied in solid foods and are therefore considered as part of the category solid foods. Typically 1 kg (modified) starch or sweetener is produced per 1 kg starch.

3) Ratio between modified starch or sweeteners and final food 0.05 kg modified starch or sweeteners/kg final food.

The most considerable applications are dairy and bakery products with a maximum modified starch/sweetener content of 5% besides the less voluminous application area of confectionary (up to 12% modified starch/sweetener). Based upon the most considerable applications and assuming ALL food (even non-bakery/non-confectionary) were to contain modified starch/sweetener, a reasonable worst-case ratio would be 0.05 kg modified starch/sweetener/kg solid food.

8.8 mg TOS/kg starch x 0.05 kg modified starch/sweeteners /kg final food =
0.44 mg TOS/L final food

HUMAN EXPOSURE ASSESSMENT

The maximum concentration of Ethyl 4 α-amylase in liquid foods is 1.06, 1.58, 5.37 and 83.1 mg TOS per liter of syrup, cereal beverage, beers, and potable alcohol, respectively. Based on application rate, knowledge of process parameters, and logical consumption patterns, the resulting theoretical exposure to α-amylase via liquid foods is highest from its use in brewing, which will be used in this risk assessment to represent a worst case scenario. In this assessment, the highest concentration of 5.37 mg TOS per liter is used to represent a worst case scenario for liquid foods.

The concentration of α-amylase in HFCS for use in solid foods is 0.44 mg TOS/kg food.

DIETARY RISK ASSESSMENT

In this assessment, the Budget method is used. This method was previously used by JECFA (FAO/WHO, 2001) and uses the following assumptions:

1) Level of consumption of foods and beverages:

For solid foods, the daily intake is set at 25 g/kg bw based on a maximum lifetime energy intake of 50 Kcal/kg bw/day. For non-milk beverages, a daily consumption of 100 ml/kg bw is used corresponding to 6 liters per day for a 60 kg adult.

2) Concentration of enzymes in foods and beverages

The concentration of enzyme in foods and beverages is the maximum application rate.

3) Proportion of foods and beverages that contain the enzymes

   a) A default of 50% of all solid foods is used to represent processed foods (i.e., 12.5 g/kg bw/day).

b) A default of 25% is used to represent non-milk beverages that may contain the enzyme (i.e., 25 ml/kg bw/day).

4) Estimation of the theoretical maximum daily intake (TMDI)

To represent a worst case scenario, TMDI for solid foods must be combined with the TMDI for beverages in the risk assessment.

Estimation of the TMDI for solid foods:

Daily intake of solid foods/starch containing enzymes = 12.5 g/kg bw/day
Maximum concentration of α-amylase in solid foods = 0.44 mg TOS/kg
TMDI/solid foods = 12.5 g food/kg bw/day x 0.44 mg TOS/kg food
= 0.0055 mg TOS/kg bw/day

Estimation of the TMDI for liquid foods

Daily intake of liquid foods containing enzymes = 25 ml/kg bw/day
Maximum concentration of α-amylase in liquid foods = 5.37 mg TOS/L
TMDI/liquid foods = 25 ml foods/kg bw/day x 5.37 mg TOS/liter food
= 0.134 mg TOS/kg bw/day

TMDI Total:

TMDI – solid foods + TMDI – liquid foods =
0.0055 mg TOS/kg bw/day + 0.134 mg TOS/kg bw/day =
0.140 mg TOS/kg bw/day

Determination of the margin of safety

The margin of safety is calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the human exposure (worst case scenario). If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{\text{NOAEL (mg/kg/day) from applicable 90-day oral tox}}{\text{Human cumulative exposure (mg/kg/day)}}$$

$$\text{Margin of Safety} = \frac{700 \text{ mg TOS/kg bw/day}}{}$$

0.140 mg TOS/kg bw/day

Margin of Safety = 5000

7.4.3 Conclusion

The proposed uses of *Geobacillus stearothermophilus* α-amylase expressed in *B. licheniformis* as a food processing aid in starch processing, brewing, cereal beverage and potable alcohol production at the maximum recommended application rates are supported by existing toxicology data. The margin of safety is calculated as 5000 based on a NOAEL of 700 mg TOS/kg bw/day (obtained from test article nearly identical to and certainly representative of α-amylase and the cumulative maximum daily exposure to α-amylase of 0.140 mg TOS/kg bw/day. Based on a margin of safety far greater than 100 even in the worst-case, the uses of α-amylase in starch processing, brewing, cereal beverage and potable alcohol production are not of human health concern.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *B. licheniformis* and enzyme preparations derived there from, including α-amylase, maltogenic α-amylase, pullulanase, subtilisin, and xylanase enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as DuPont Industrial Biosciences' own published and unpublished safety studies and GRAS determinations, support such a conclusion.

B. licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

Analysis of the safety based on Pariza and Johnson decision tree indicates that *G. stearothermophilus* α-amylase expressed in *B. licheniformis* is accepted, even without new toxicology data (See section 7).

Based on the available data from the literature and generated by DuPont Industrial Biosciences, the company has concluded that α-amylase from *B. licheniformis (*BML612-Ethyl-4-CAP75) is safe and suitable for use in the grain and tuber starch processing, brewing, cereal beverage manufacture and potable alcohol. The GRAS determination was reviewed by Dr. Michael Pariza who concurred with DuPont's determination that the enzyme is GRAS for its intended uses (Appendix 6).

9. LIST OF APPENDICES

Appendix 1. 21CFR170.30

Appendix 2. Amino Acid Sequence of Ethyl 4 α-amylase

Appendix 3. Alpha-amylase production process

Appendix 4. Certificates of Analysis, 3 representative lots

Appendix 5. *Bacillus licheniformis* safe strain lineage and toxicology summary

Appendix 6. GRAS Concurrence Letter from Dr. Pariza

10. LIST OF REFERENCES

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Appendix 1: 21 CFR 170.30

[Code of Federal Regulations]

[Title 21, Volume 3]

[Revised as of April 1, 2005]

From the U.S. Government Printing Office via GPO Access

[CITE: 21CFR170.30]

[Page 13-15]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN

SERVICES (CONTINUED)

PART 170_FOOD ADDITIVES--Table of Contents

Subpart B_Food Additive Safety

Sec. 170.30 Eligibility for classification as generally recognized as safe (GRAS).

(a) General recognition of safety may be based only on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.

(b) General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

(c)(1) General recognition of safety through experience based on common use in food prior to January 1, 1958, may be determined without the quantity or quality of scientific procedures required for approval of a food additive regulation. General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information. An ingredient not in common use in food prior to January 1, 1958, may achieve general recognition of safety only through scientific procedures.

(2) A substance used in food prior to January 1, 1958, may be generally recognized as safe through experience based on its common use in food when that use occurred exclusively or primarily outside of the United States if the information about the experience establishes that the use of the substance is safe within the meaning of the act (see Sec. 170.3(i)). Common use in food prior to January 1, 1958, that occurred outside of the United States shall be documented by published or other information and shall be corroborated by information from a second, independent source that confirms the history and circumstances of use of the substance. The information used to document and to corroborate the history and circumstances of use of the substance must be generally available; that is, it must be widely available in the country in which the history of use has occurred and readily available to interested qualified experts in this country. Persons claiming GRAS status for a substance based on its common use in food outside of the United States should obtain FDA concurrence that the use of the substance is GRAS.

(d) The food ingredients listed as GRAS in part 182 of this chapter or affirmed as GRAS in part 184 or Sec. 186.1 of this chapter do not include all substances that are generally recognized as safe for their intended use in food. Because of the large number of substances the intended use of which results or may reasonably be expected to result, directly or indirectly, in their becoming a component or otherwise affecting the characteristics of food, it is impracticable to list all such substances that are GRAS. A food ingredient of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effects, which is subject only to conventional processing as practiced prior to January 1, 1958, and for which no known safety hazard exists, will ordinarily be regarded as GRAS without specific inclusion in part 182, part 184 or Sec. 186.1 of this chapter.

(e) Food ingredients were listed as GRAS in part 182 of this chapter during 1958-1962 without a detailed scientific review of all available data and information relating to their safety. Beginning in 1969, the Food and Drug Administration has undertaken a systematic review of the status of all ingredients used in food on the determination that they are GRAS or subject to a prior sanction. All determinations of GRAS status or food additive status or prior sanction status pursuant to this review shall be handled pursuant to Sec. Sec. 170.35, 170.38, and 180.1 of this chapter. Affirmation of GRAS status shall be announced in part 184 or Sec. 186.1 of this chapter.

(f) The status of the following food ingredients will be reviewed and affirmed as GRAS or determined to be a food additive or subject to a prior sanction pursuant to Sec. 170.35, Sec. 170.38, or Sec. 180.1 of this chapter:

(1) Any substance of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effect, for which no health hazard is known, and which has been modified by processes first introduced into commercial use after January 1, 1958, which may reasonably be expected significantly to alter the composition of the substance.

(2) Any substance of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effect, for which no health hazard is known, that has had significant alteration of composition by breeding or selection after January 1, 1958, where the change may be reasonably expected to alter the nutritive value or the concentration of toxic constituents.

(3) Distillates, isolates, extracts, and concentration of extracts of GRAS substances.

(4) Reaction products of GRAS substances.

(5) Substances not of a natural biological origin, including those for which evidence is offered that they are identical to a GRAS counterpart of natural biological origin.

(6) Substances of natural biological origin intended for consumption for other than their nutrient properties.

(g) A food ingredient that is not GRAS or subject to a prior sanction requires a food additive regulation promulgated under section 409 of the act before it may be directly or indirectly added to food.

(h) A food ingredient that is listed as GRAS in part 182 of this chapter or affirmed as GRAS in part 184 or Sec. 186.1 of this chapter shall be regarded as GRAS only if, in addition to all the requirements in the applicable regulation, it also meets all of the following requirements:

(1) It complies with any applicable food grade specifications of the Food Chemicals Codex, 2d Ed. (1972), or, if specifically indicated in the GRAS affirmation regulation, the Food Chemicals Codex, 3d Ed. (1981), which are incorporated by reference, except that any substance used as a component of articles that contact food and affirmed as GRAS in Sec. 186.1 of this chapter shall comply with the specifications therein, or in the absence of such specifications, shall be of a purity suitable for its intended use. Copies may be obtained from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-

741-6030, or go to: http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html.

(2) It performs an appropriate function in the food or food-contact article in which it is used.

(3) It is used at a level no higher than necessary to achieve its intended purpose in that food or, if used as a component of a food-contact article, at a level no higher than necessary to achieve its intended purpose in that article.

(i) If a substance is affirmed as GRAS in part 184 or Sec. 186.1 of this chapter with no limitation other than good manufacturing practice, it shall be regarded as GRAS if its conditions of use are not significantly different from those reported in the regulation as the basis on which the GRAS status of the substance was affirmed. If the conditions of use are significantly different, such use of the substance may not be GRAS. In such a case a manufacturer may not rely on the regulation as authorizing the use but must independently establish that the use is GRAS or must use the substance in accordance with a food additive regulation.

(j) If an ingredient is affirmed as GRAS in part 184 or Sec. 186.1 of this chapter with specific limitation(s), it may be used in food only within such limitation(s) (including the category of food(s), the functional use(s) of the ingredient, and the level(s) of use). Any use of such an ingredient not in full compliance with each such established limitation shall require a food additive regulation.

(k) Pursuant to Sec. 170.35, a food ingredient may be affirmed as GRAS in part 184 or Sec. 186.1 of this chapter for a specific use(s) without a general evaluation of use of the ingredient. In addition to the use(s) specified in the regulation, other uses of such an ingredient may also be GRAS. Any affirmation of GRAS status for a specific use(s), without a general evaluation of use of the ingredient, is subject to reconsideration upon such evaluation.

(l) New information may at any time require reconsideration of the GRAS status of a food ingredient. Any change in part 182, part 184, or Sec. 186.1 of this chapter shall be accomplished pursuant to Sec. 170.38.

[42 FR 14483, Mar. 15, 1977, as amended at 49 FR 5610, Feb. 14, 1984; 53

FR 16546, May 10, 1988]

Appendix 2: Amino Acid Sequence of Ethyl 4 α-amylase

AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGTSRSDVGYGVYDLYDLGEF
NQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQI
QAWTKFDFPGRGNTYSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLMYAD
LDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSYVRSQTGKPLFTVGEYWSYDINK
LHNYITKTNGTMSLFDAPLHNKFYTASKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSW
VDPWFKPLAYAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQHDYLDHSDIIGWTR
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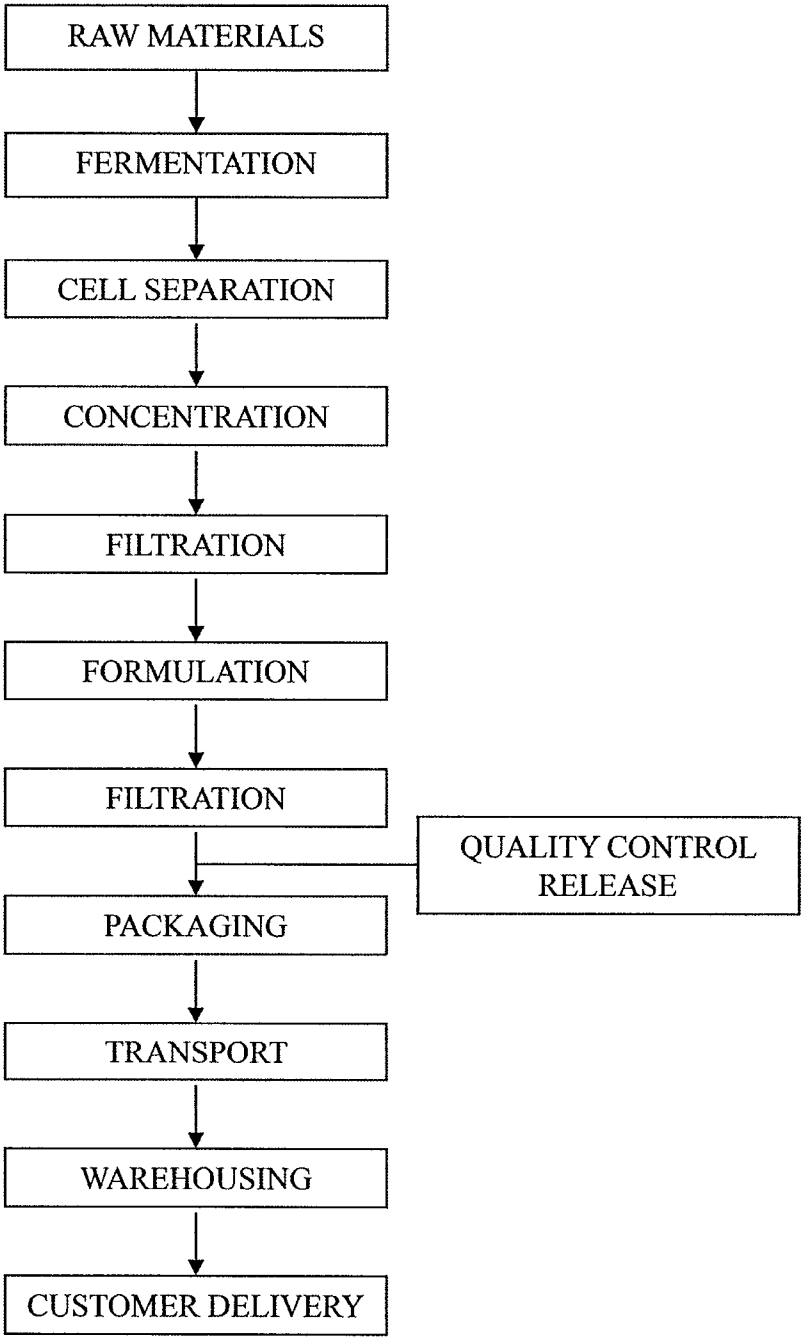
Appendix 3: Production Process



Appendix 4: Certificates of Analysis, 3 representative lots

CERTIFICATE OF ANALYSIS

PRODUCT: **Ethyl 4 alpha-amylase**

LOT NUMBER: **7202367061**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Alpha-amylase	AAU/g	13400 - 14600	14390
MICROBIOLOGICAL ANALYSIS			
Coliforms	CFU/ml	0 - 30	<10
E. coli	/25ml	Negative	Negative
Salmonella	/25ml	Negative	Negative
Production strain	/ml	Negative	Negative
Antibacterial activity	/ml	Negative	Negative
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<0.1

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

24-June-2015 Damien Cordero
Date QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **Ethyl 4 alpha-amylase**

LOT NUMBER: **7202429437**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Alpha-amylase	AAU/g	13400 - 14600	14575
MICROBIOLOGICAL ANALYSIS			
Coliforms	CFU/ml	0 - 30	<10
E. coli	/25ml	Negative	Negative
Salmonella	/25ml	Negative	Negative
Production strain	/ml	Negative	Negative
Antibacterial activity	/ml	Negative	Negative
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<0.1

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

24-June-2015 Damien Cordero
Date QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **Ethyl 4 alpha-amylase**

LOT NUMBER: **7202424356**

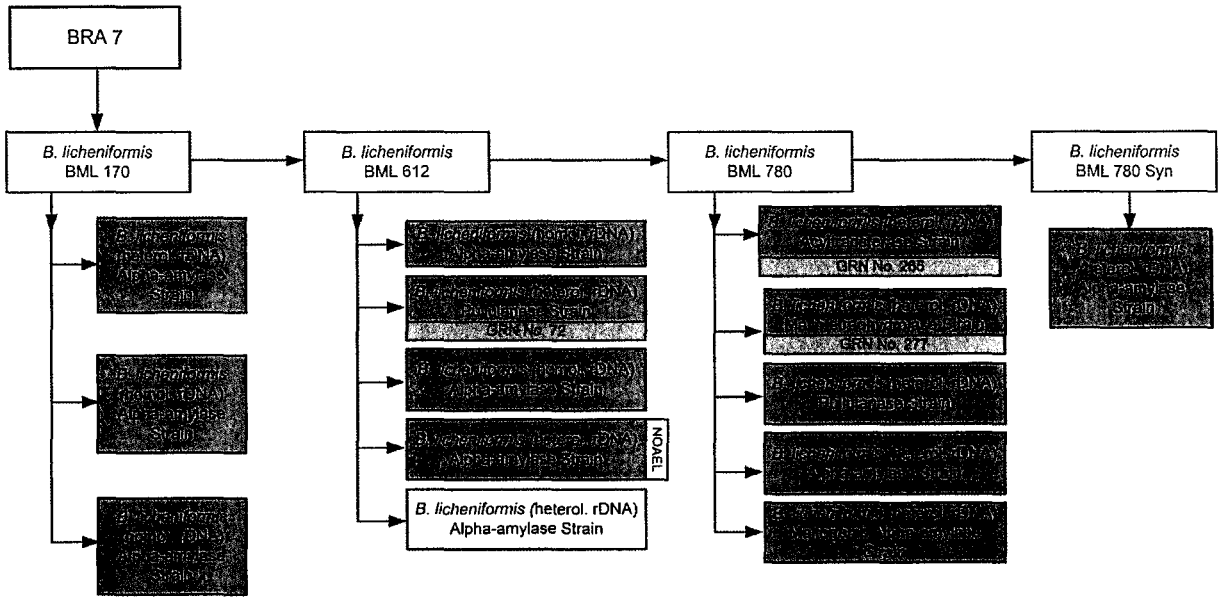
ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Alpha-amylase	AAU/g	13400 - 14600	14468
MICROBIOLOGICAL ANALYSIS			
Coliforms	CFU/ml	0 - 30	<10
E. coli	/25ml	Negative	Negative
Salmonella	/25ml	Negative	Negative
Production strain	/ml	Negative	Negative
Antibacterial activity	/ml	Negative	Negative
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<0.1

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

24-June-2015 Damien Cordero
Date QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix 5: *Bacillus licheniformis* safe strain lineage and toxicology summary



All commercial enzymes derived from this Safe Strain Lineage were determined to be GRAS, with GRAS Notices reviewed by the US FDA for enzymes from strains designated with green horizontal banners (indicating the GRAS Notice number).

The subject strain is the alpha-amylase producing strain highlighted in yellow.

The safety of this alpha-amylase is fully supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage.

According to the Safe Strain Lineage concept, the NOAEL for the alpha-amylase from the closely related production strain is used to support the safety of the subject alpha-amylase in the intended use, as indicated with yellow flag labeled "NOAEL".

A Determination of Safe Strain Lineage for *Bacillus licheniformis* host strain BRA7

The species *Bacillus licheniformis* has been used as a production organism for enzymes by DuPont Industrial Biosciences (legacy Genencor), since 1989.

Genencor has conducted numerous toxicology and genotoxicity studies with enzyme preparations derived from various *Bacillus licheniformis* strains derived from *Bacillus licheniformis* host strain BRA7. An evaluation and summary of the data are discussed in this memorandum. All toxicology studies sponsored by Genencor strictly follow corresponding OECD guidelines and are conducted in compliance with all current Good Laboratory Practice Standards. A summary table of the toxicology studies can be found in Figure 1.

All the enzymes discussed below have been evaluated by GRAS panels who have determined that the enzymes are safe for their intended uses and are Generally Recognized As Safe (GRAS).

A. Enzymes derived from *Bacillus licheniformis* BML 170

A.1. Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

A battery of genotoxicity assays was conducted and under the conditions of these assays, the AA enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in the presence and absence of metabolic activation. The potential of the enzyme to induce systemic toxicity was investigated after repeated daily oral administration of the ultra-filtered concentrate of the product in Wistar rats of both sexes. The enzyme was given by gavage for 28 consecutive days at 0, 20, 100 or 500 mg/kg body. Under the conditions of this study, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 500 mg /kg bw/day.

A.2. Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

A battery of genotoxicity assays was conducted and under the conditions of these assays, the AA enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in the presence and absence of metabolic activation. The systemic toxicity potential of the enzyme has not been investigated, but was not expected to be different from the AA enzyme in A.1 above.

A.3. Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

This enzyme is a low pH α-amylase produced by a variant of an alpha-amylase (homol. rDNA) strain. The genotoxicity potential of the enzyme was investigated in a bacterial reverse mutation assay (Ames assay) and a chromosomal aberration assay with human lymphocytes. The enzyme was not a mutagen or clastogen in both the presence and absence of metabolic activity. The potential toxicity after oral administration (gavage) was investigated in the rat for 13 consecutive weeks. Groups of animals received 0, 625, 1250 or 2,500 mg/kg/day of the ultra-filtered concentrate corresponding to 29.25, 58.50 and 117 mg TOS/kg/day. No treatment related adverse effects were noted in this study and the NOAEL was established at the highest dose tested, 2,500 mg/kg/day or 117 mg TOS/kg/day.

References

Bio-Research Laboratories, Inc.: 13-week gavage subchronic toxicity study. Final report No. 87629, December 10, 1996.
Microbiological Associates, Inc.: *In vitro* chromosomal aberrations. Final report NO. G96B072.346, February 20, 1997.
Microbiological Associates, Inc.: Bacterial reverse mutation assay. Final report NO. G96B072.502, December 13, 1996.

B. Products derived from *Bacillus licheniformis* BML 612

B.1. Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

This enzyme is a low pH α amylase produced from a *Bacillus licheniformis* (homol. rDNA) strain. The mutagenic potential of the enzyme was investigated in a bacterial reverse mutation assay (Ames assay) and an *in vitro* chromosomal aberration assay with human peripheral lymphocytes. Under the conditions of these assays, the enzyme was not a mutagen or clastogen in both the presence and absence of metabolic activation. The systemic toxicity potential was investigated in male and female rats treated with the enzyme for 13 consecutive weeks. The ultra-filtered concentrate was given by oral gavage to groups of rats at 0, 625, 1,250 or 2,500 mg/kg/day. There were no treatment related effects. The NOAEL was established at the highest dose tested, 2,500 mg/kg/day.

References

Harlan Laboratories: Acute oral toxicity in the rat – Fixed dose method. Report No. 2420/0016, June 01, 2009.
Harlan Laboratories: Acute inhalation toxicity (nose only) in the rat. Report No. 2420/0017, July 15, 2009.
Harlan Laboratories: Acute dermal irritation in the rabbit. Report No. 2420/0018, June 01, 2009.
Harlan Laboratories: Acute eye irritation in the rabbit. Report No. 2420/0019, June 01, 2009.
Harlan Laboratories: Local lymph node assay in the mouse. Report No. 2420/0020, August 05, 2009.
Harlan Laboratories: *Salmonella typhimurium* and *Escherischia coli* reverse mutation assay. Report No. 2420/0021, May 15, 2009.
Harlan Laboratories: Chromosome aberration test in human lymphocytes *in vitro*. Report No. 2420/0022, August 7, 2009.
Harlan Laboratories: 90-day repeated oral (gavage) toxicity study in the rat. Report No. 2420/0023, October 5, 2009

B.2. Pullulanase from *Bacillus licheniformis* (heterol. rDNA) strain

This enzyme is a pullulanase enzyme produced by a *Bacillus licheniformis* (homol. rDNA) strain with applications in foods and its safety has been investigated. Pullulanase was not an irritant to the eyes and skin. Pullulanase was practically non-toxic based on acute inhalation and acute ingestion studies. In

genotoxicity studies, Pullulanase was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogenic or an aneugen in an *in vitro* chromosomal aberration assay with human

peripheral lymphocytes in both the presence and absence of metabolic activation. Daily oral (gavage) administration of ultra-filtered concentrate of Pullulanase for 90 consecutive days up to and including a dose level of 2,500 mg/kg did not result in any treatment-related adverse effects in rats. A NOAEL (no observed adverse effect level) was established at 2,500 mg/kg/day of the UF concentrate. Based on a specific gravity of 1.034, a total protein of 69.79 mg/ml and a total organic solid content of 9.82%, this NOAEL (2,500 mg/kg/day) corresponds to 168.9 mg total protein/kg/day or 237.64 mg TOS/kg/day.

References

BioReliance No. AA16GE.507.BTL, Bacterial reverse mutation assay with an independent repeat assay, August 1999.
BioReliance No. AA16GE.341.BTL, *In vitro* mammalian chromosome aberration test, September 1999.
ClinTrials BioResearch No. 88873, A 13-week oral gavage toxicity study of Pullulanase in the albino rats, August 1999.
IRDC No. 713-002, 4-week dietary toxicity study in rats with Pullulanase, June 1994.
IRDC No. 713-003, Primary dermal irritation test in rabbits with Pullulanase, February 1994.
IRDC No. 713-004, Primary eye irritation study in rabbits with Pullulanase, February 1994.
IRDC No. 713-005, Acute inhalation toxicity evaluation in rats with Pullulanase, April 1994.
IRDC No. 713-006, Bacterial reverse mutation assay (Ames assay) with Pullulanase, Feb 1994 (Genesys Final Report No. 93027, February 1994).
IRDC No. 713-007, *In vitro* forward mutation assay using the L5178Y/tk+/- mouse lymphoma cells with Pullulanase, Feb 1994 (Genesys Final Report No. 93028, February 1994).
IRDC No. 713-009, *In vivo* mouse bone marrow chromosome aberration test with Pullulanase, August 1994 (Genesys Final report No. 93030, August 1994).

B.3 Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

The safety of the α-amylase enzyme produced from a *Bacillus licheniformis* (homol. rDNA) strain was assessed in a battery of toxicology studies investigating its acute oral, inhalation, irritation, skin sensitization, mutagenic and systemic toxicity potential. The enzyme was not an eye or skin irritant and was not acutely toxic by ingestion. It is not a dermal sensitizer based on the results of the local lymph node assay. A battery of genotoxicity assays was conducted and under the conditions of these assays and was determined not to be a mutagen in the bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in the *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. Daily administration of the enzyme's ultra-filtered concentrate by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL was established at the highest dose tested, 80 mg total protein/kg bw/day corresponding to 110 mg TOS/kg bw/day.

References

Covance Laboratories: 13-week gavage sub-chronic toxicity study with alpha amylase. Final report No. 7043-100, December 7, 1999.
MA BioServices Inc.: In vitro mammalian chromosome aberration test with alpha amylase. Final report No. G98AG08.341, June 12, 1998.
MA BioServices Inc.: Bacterial reverse mutation assay with alpha amylase. Final report NO.

G98AG08.507, August 27, 1998.

B.4 Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The AA enzyme was not mutagenic in the Ames assay and was not clastogenic in the mammalian system (*in vitro* chromosomal aberration assay with human peripheral lymphocytes) in both the presence and absence of metabolic activation. The systemic toxicity after repeated daily oral administration (gavage) of the ultra-filtered concentrate was investigated in Sprague Dawley rats of both sexes for 90 consecutive days at 0, 16, 32, or 64 mg total protein/kg body weight. These doses corresponded to 0, 175, 350 or 700 mg TOS/kg bw/day, respectively. There were no treatment-related effects in this study. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 64 mg total protein/kg bw/day or 700 mg TOS/kg bw/day.

References

Scantox Study No. 57860, Acute dermal irritation study in the rabbit with Alpha Amylase, April 20, 2005.
Scantox Study No. 57861, Ocular irritation test in the rabbit with Alpha Amylase, March 8, 2005.
Scantox Study No. 57831, Ames Test with Alpha Amylase, April 14, 2005.
Scantox Study No. 57832, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, Alpha Amylase, August 15 2005.
Scantox Study No. 58136, A 13-week oral (gavage) toxicity study in rats with Alpha Amylase, June 24, 2005.

C. Products derived from *Bacillus licheniformis* BML 780

C.1. Acyltransferase from *Bacillus licheniformis* (heterol. rDNA) strain

Acyltransferase's safety was assessed in a battery of toxicology studies. The enzyme was not an irritant to the eyes and skin and was practically non-toxic based on an acute oral ingestion study. In genotoxicity studies, the enzyme was not mutagenic in the bacterial reverse mutation assay (Ames assay), was not clastogenic or aneugenic in the *in vitro* chromosomal aberration assay with human peripheral lymphocytes, and was not aneugenic in an *in vivo* mouse micronucleus assay in both the presence and absence of metabolic activation. The potential systemic toxicity of the enzyme after repeated daily oral administration of the ultra-filtered concentrate was investigated in SPF Sprague Dawley rats for 90 consecutive days. Groups of rats of both sexes were gavaged daily with 0, 4.56, 13.68 or 41.00 mg total protein/kg body weight corresponding to 0, 13.0, 39.0 and 116.9 mg TOS/kg bw/day, respectively. Daily oral administration of the enzyme up to and including a dose level of 41 mg total protein/kg bw/day did not result in any manifestation of adverse health effects. A NOAEL was established at 41 mg total protein or 116.9 mg TOS/kg bw/day.

References

Scantox Study No. 62125, Acute dermal irritation study in the rabbit with Acyltransferase, September 2006.
Scantox Study No. 62124, Acute eye irritation/corrosion study in the rabbit with Acyltransferase, September 2006.
Scantox Study No. 62123, Acute oral toxicity study in the rat with Acyltransferase. September 2006.

Scantox Study No. 62127, Acyltransferase, Ames Test, October 2006.
Scantox Study No. 62126, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, Acyltransferase, 2006.
Scantox Study No. 64415, Mouse micronucleus test with Acyltransferase, November 2006.
Scantox Study No. 62129, A13-week oral (gavage) toxicity study in rats with Acyltransferase, October 2006.

C.2. Maltotetraohydrolase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of the maltotetraohydrolase produced by a *Bacillus licheniformis* (heterol. rDNA) strain that was assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. The enzyme was not a skin irritant, was not acutely toxic by ingestion and is a mild eye irritant. A battery of genotoxicity assays was conducted and under the conditions of these assays, the enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. The potential of the maltotetraohydrolase amylase to induce systemic toxicity after repeated daily oral (gavage) administration was investigated in Wistar rats of both sexes. Ultra-filtered enzyme concentrate was given for 90 consecutive days by gavage at 0, 23.7, 47.4 or 79 mg total protein/kg body weight corresponding to 0, 27.3, 54.5 or 90.9 mg TOS/kg bw/day, respectively. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 79 mg total protein/kg bw/day corresponding to 90.0 mg TOS/kg bw/day.

References

SafePharm Lab Study No. 2420/0005, Acute dermal irritation study in the rabbit with maltotetraohydrolase, 15 April 2008.
SafePharm Lab Study No. 2420/0004, Acute eye irritation/corrosion study in the rabbit with maltotetraohydrolase, 28 April 2008.
SafePharm Lab Study No. 2420/0003, Acute oral toxicity study in the rat with maltotetraohydrolase, fixed dosed method, 13 May 2008.
SafePharm Lab Study No. 2420/0006, Reverse mutation assay – Ames Test with maltotetraohydrolase, 12 June 2008.
SafePharm Lab Study No. 2420/0007, Chromosome aberration test in human *lymphocytes in vitro* with maltotetraohydrolase, 06 June 2008.
SafePharm Lab Study No. 2420/0008, 90 day repeated oral (gavage) toxicity study in the rat with maltotetraohydrolase, 14 October 2008.

C.3. Pullulanase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of Truncated PU is assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays Truncated PU is not a mutagen or clastogen. Daily administration of Truncated PU by gavage for 90 continuous days did not result in overt signs of systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 500 mg

TOS/kg bw/day corresponding to 260 mg TP/kg bw/day.

References

BioReliance: H-30648: Bacterial reverse mutation assay; Report No. AD69TA.507001.BTL; Dupont No. 20265-513; Final report dated July 22, 2013.
BioReliance: H-30648: *In vitro* mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. AD69TA.341.BTL; Dupont No. 20265-544; Final report dated July 30, 2013.
Dupont Haskell Global Centers: H-30648 Subchronic toxicity 90 day gavage study in rats; Report No. 20265-1026; Final report dated February 6, 2014.

C.4. Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of the AA enzyme was assessed in a battery of toxicology studies investigating its irritation, acute oral, genotoxic and systemic toxicity potential. The enzyme was not an eye or skin irritant. Genotoxicity assays were conducted and under the conditions of these assays, the enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. The systemic toxicity was investigated in SPF Sprague Dawley rats. Ultra-filtered concentrate was given by gavage daily for 90 consecutive days at 0, 4.96, 12.4 and 37.2 mg total protein/kg bw corresponding to 0, 8.9, 22.27 and 66.81 mg TOS/kg bw/day, respectively. Daily administration of GC 358 by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL was established at 37.2 mg total protein/kg bw/day corresponding to 66.81 mg TOS/kg bw/day.

References

Harlan Laboratories No. 41100560: Alpha-amylase, Acute dermal irritation in the rabbit, June 10, 2011.
Harlan Laboratories No. 41100561: Alpha-amylase, Acute eye irritation in the rabbit, July 14, 2011.
Harlan Laboratories No. 41100559: Alpha-amylase, Acute oral toxicity in the rat – Fixed dose method, July 18, 2011.
Harlan Laboratories No. 41100562: Alpha-amylase, Reverse mutation assay "Ames Test" using *Salmonella typhimurium* and *Escherichia coli*, September 7, 2011.
Harlan Laboratories No. 41100563: Alpha-amylase, Chromosome aberration test in human lymphocytes *in vitro*, September 16, 2011.
Harlan Laboratories No. 41100564: Ninety day repeated dose oral (gavage) toxicity study in the rat – Alpha-amylase, December 6, 2011.

C.5. Maltogenic Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of the maltogenic alpha-amylase was assessed in a battery of toxicology studies investigating its dermal and eye irritation, acute oral, genotoxic and systemic toxicity potential. Maltogenic alpha-amylase was not an eye or skin irritant. Genotoxicity assays were conducted and under the conditions of these assays Maltogenic alpha-amylase was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an in vitro chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. The systemic toxicity

of Maltogenic alpha-amylase was investigated in Wistar rats. Ultra-filtered concentrate of Maltogenic alpha-amylase was given by gavage daily for 90 consecutive days at 0, 13.9, 27.8, and 55.6 mg total protein/kg bw corresponding to 0, 20, 40, and 80 mg TOS/kg bw/day, respectively. Daily administration of Maltogenic alpha-amylase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL was established at 55.6 mg total protein/kg bw/day corresponding to 80 mg TOS/kg bw/day.

References

BioReliance: H-30648: Bacterial reverse mutation assay; Report No. AD69TA.507001.BTL; Dupont No. 20265-513; Final report dated July 22, 2013.
BioReliance: H-30648: *In vitro* mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. AD69TA.341.BTL; Dupont No. 20265-544; Final report dated July 30, 2013.
Dupont Haskell Global Centers: H-30648 (Truncated PU) Subchronic toxicity 90-day gavage study in rats; Report No. 20265-1026; Final report dated February 6, 2014.

D. Products derived from *Bacillus licheniformis* BML 780 syn

D.1. Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of Alpha amylase (C16F UFC) is assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays Alpha amylase (C16F UFC) is not a mutagen or clastogen. Daily administration of Alpha amylase (Level 10 UFC) by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 500 mg TOS/kg bw/day (corresponding to 272 mg TP/kg bw/day).

References

BioReliance: H-30929: Bacterial reverse mutation assay; Report No. AD84GP.507001.BTL; Dupont No. 20558-513; Final report dated February 04, 2014.
Dupont Haskell Global Centers: H-30929: *In vitro* mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. 20558-544; Final report dated February 21, 2014.
MPI Research: H-30929: Subchronic toxicity 90 day oral gavage study in rats; Report No. 125-180; Final report dated October 2014.

SUMMARY

Acute toxicity and Irritation Studies
All enzyme preparations produced from various strains of *Bacillus licheniformis* are practically non-toxic by ingestion (oral LD_{50} greater than 2000 mg/kg) and are not irritating to the skin or eyes.

Genotoxicity
Numerous genotoxicity studies were conducted and all enzyme preparations produced from various

strains of *Bacillus licheniformis* are not mutagenic, not aneugenic and not clastogenic.

Systemic Toxicity

A review of all repeated oral administration studies in rodents suggests that no specific target organ toxicity can be identified with enzyme preparations produced from various strains of *Bacillus licheniformis*. There were no adverse effects on body weight, feed and water consumption and daily clinical observations. There were no effects on ophthalmologic examination, hematology, clinical chemistry, urinalysis and functional observation battery. At necropsy, there was no specific target organ toxicity that can be attributed to these enzyme preparations.

DISCUSSION

The safety of enzyme preparations produced from various strains of *Bacillus licheniformis* was investigated for their potential irritation, genotoxicity and systemic toxicity in studies designed following OECD guidelines. Studies investigating the systemic toxicity of enzymes from *B. licheniformis* were designed to follow the OECD Guideline No. 408 (Sub-chronic oral toxicity – Rodent: 90 day study) (adopted 21 September 1998) and the EPA Guideline OPPTS 870.3100 (August 1998). Studies investigating the genotoxic potential were designed to follow the OECD Guideline No. 471 (Bacterial reverse mutation assay) (May 30, 2008) and Guideline No. 473 (Chromosome Aberration Assay) (May 30, 2008). OECD Guideline No. 429 (Skin sensitization: Local lymph node assay) (April 24, 2002) was used to detect the potential for skin sensitization. All studies sponsored by DuPont Industrial Biosciences (legacy Genencor) were performed in compliance with all current Good Laboratory Practice Standards.

A review of all toxicology studies conducted with enzyme preparations produced by different strains of *Bacillus licheniformis* indicates that, regardless of the production organism strain, all enzyme preparations are not irritating to the skin and eyes, are not skin sensitizers, are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ. The NOAEL obtained from the oral (gavage) administration studies was always the highest dose tested. Thus, the existing data substantiate and demonstrate that the *Bacillus licheniformis* host strain BRA7 lineage is indeed a safe strain lineage and all enzyme preparations produced by these *Bacillus licheniformis* strain are safe and suitable for their intended uses. Due to the consistency of the findings from enzyme preparations derived from different *Bacillus licheniformis* host strain BRA7 derived strains, it is expected that any new enzyme preparation produced using the *Bacillus licheniformis* host strain BRA7 lineage would behave similarly from a toxicological standpoint. Therefore, it can be concluded that Genencor can utilize this *Bacillus licheniformis* host strain BRA7 safe strain lineage to produce other enzymes without conducting new toxicology and/or safety studies to demonstrate their safety.

TABLE 1

SUMMARY OF TOXICOLOGY DATA FROM ENZYME PREPARATIONS PRODUCED FROM DIFFERENT *BACILLUS LICHENIFORMIS* STRAINS

***BACILLUS LICHENIFORMIS* host strain BRA7 STRAIN LINEAGE**													
	HOST STRAIN BML170			**HOST STRAIN BML612**				**HOST STRAIN BML780**					**HOST STRAIN BML 780 Syn**
Enzyme	α-amylase	α-amylase	α-amylase	α-amylase	Pullulanase	α-amylase	α-amylase	Acyl Transferase	α-amylase	Maltotetrao-hydrolase	Maltogenic α-amylase	Pullulanase	α-amylase
Genotoxicity	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects
Systemic Toxicity	None	No Data	None	None	None	None	None	None	None	None	None	None	None
NOAEL (TOS/kg/day)		No Data	117 mg	420.75 mg	237.64 mg	700 mg	110 mg	116.9 mg	90 mg	66.81 mg	80 mg	500 mg	500 mg
NOAEL (total protein/kg/d)				280.75 mg	168.9 mg	64 mg	80 mg	41 mg	79 mg	37.2 mg	55.6 mg	260 mg	272 mg
NOAEL (UFC/kg/d)	500 mg/kg/d (28-day oral)		2500 mg/kg/d	2500 mg/kg/d									

Appendix 6: GRAS Concurrence letter from Dr. Pariza

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Michael W. Pariza, Member

July 25, 2015

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
DuPont Industrial Biosciences
Genencor / DuPont US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS opinion on the uses of Genencor/DuPont's α-amylase Ethyl 4 AA expressed by *Bacillus licheniformis* BML612-Ethyl-4-CAP75 for grain and tuber starch processing, brewing, cereal beverage manufacture, as well as in potable alcohol and fuel ethanol with resulting co-products (corn gluten feed/meal, brewers' grains, distillers' grains) used as animal feed

Dear Dr. Sewalt,

I have reviewed the information that you provided on Genencor/DuPont's Ethyl 4 AA α-amylase preparation, produced by *Bacillus licheniformis* BML612-Ethyl-4-CAP75 (GICC03266) that has been genetically engineered to express the α-amylase enzyme from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*). The intended uses of the Ethyl 4 AA α-amylase are in grain and tuber starch processing, brewing, cereal beverage manufacture, as well as in potable alcohol and fuel ethanol with resulting co-products (corn gluten feed/meal, brewers' grains, distillers' grains) used as animal feed. In these applications Ethyl 4 AA α-amylase will be used as a processing aid where the enzyme is either not present in the final food/feed or present as inactive protein in insignificant quantities having no function or technical effect in the final food/feed.

In evaluating the Ethyl 4 AA α-amylase product, I considered the biology of *B. licheniformis* and the gene donor, *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*); information that you provided on the Ethyl 4 AA gene and α-amylase protein structure including its similarity to other α-amylases that have histories of safe use in food manufacture; the construction of *B. licheniformis* BML612-Ethyl-4-CAP75 (GICC03266); information that you previously provided on other closely

related Genencor/DuPont's amylase enzymes; and other pertinent information that is available in the peer-reviewed scientific literature.

Bacillus licheniformis is a common soil microorganism that has not been associated with pathogenicity or toxigenicity for humans or other animals. This species is listed in the Food Chemicals Codex as a source of carbohydrase and protease enzyme preparations used in food processing. The FDA has affirmed that a mixed carbohydrase and protease enzyme product derived from *B. licheniformis* is GRAS for use in the production of certain foods (21 CFR 184.1027). GRP 5G0415 (converted to GRN 000072) cites published reports on the cloning and expression of proteins in *B. licheniformis* for use in food products, and FDA issued a 'no objection' letter for the uses of *B. deramificans* pullulanase expressed by *B. licheniformis* GICC03088 as described in GRN 000072.

Genencor and its parent companies have developed a lineage of safe enzyme production strains from *B. licheniformis* Bra7, a classical industrial strain developed from its wild-type parent via classical strain improvement methodologies. *Bacillus licheniformis* GICC03088, described in GRN 000072, is a member of this safe strain lineage of *B. licheniformis* Bra7 enzyme production strains, and *B. licheniformis* BML612-Ethyl-4-CAP75 (GICC03266), which is the subject of this GRAS opinion, is another. *Bacillus licheniformis* Bra7 and strains derived from it have been used to produce α-amylase since 1989.

Geobacillus stearothermophilus (formerly *Bacillus stearothermophilus*) is a non-pathogenic, non-toxigenic thermophile that is commonly found in soil, lake/ocean sediments, and hot springs. It is regarded as a food spoilage organism and has not been associated with pathogenicity or toxigenicity for humans or other animals. The donor strain used as a source for the α-amylase gene was *G. stearothermophilus* ASP-154, an asporogenic mutant of *G. stearothermophilus* 55-9C6, that is deposited in the American Type strain Culture Collection (ATCC) as *B. stearothermophilus* ATCC 39709.

The Ethyl 4 AA enzyme is 100% homologous to the native *G. stearothermophilus* α-amylase enzyme, which has a long history of safe use as a food ingredient and was affirmed as GRAS (21CFR184.1012). The gene for the native *G. stearothermophilus* α-amylase was truncated during the genetic construction procedure but this change did not alter the amino acid sequence of the final enzyme product. The amino acid sequence of the Ethyl 4 AA enzyme protein was subjected to a BLAST analysis; no homology to known toxin sequences was detected.

Given that the introduced DNA was fully characterized and shown to be free of sequences associated with safety concern, safety evaluation for the Ethyl 4 AA α-amylase preparation was based *in vitro* and *in vivo* studies conducted on the EBS2 α-amylase preparation. The EBS2 α-amylase is the same wild-type α-amylase as Ethyl 4 AA except for 2 amino acid deletions, and is produced by *B. licheniformis* BML 612-EBS2c1 (GICC03191), a closely related strain within the safe lineage of *B. licheniformis* Bra7. The safety evaluation studies for the EBS2 α-amylase included a sub-chronic (90) day Sprague-Dawley rat feeding trial, from which NOAELs (No Observed Adverse Effect Levels) were established from the highest dose tested (5% of the diet). The margins of safety from all uses were then determined to be as follows: for human exposure, 5000; for cattle, pigs and poultry, respectively, 446, 356, and 412. These values are all well above the typical 100-fold safety factor that is traditionally used for food ingredients.

Construction of the production strain, *B. licheniformis* BML612-Ethyl-4-CAP75 (GICC03266), which expresses the truncated native *G. stearothermophilus* α-amylase gene and produces the Ethyl 4 AA α-amylase enzyme, utilized methods and reagents that are appropriate for food/feed grade ingredient production strains. The manufacturing process including the ingredients used for fermentation, extraction and concentration of the Ethyl 4 AA α-amylase preparation, and the specifications for the Ethyl 4 AA α-amylase preparation, are appropriate for food/feed grade ingredients.

Based on the foregoing, I concur with your conclusion that the *B. licheniformis* BML612-Ethyl-4-CAP75 (GICC03266) production strain, which expresses the truncated native *G. stearothermophilus* α-amylase gene and produces the Ethyl 4 AA enzyme which is 100% homologous to the native *G. stearothermophilus* α-amylase protein that has been affirmed as GRAS (21CFR184.1012), is safe to use for the manufacture of food/feed grade α-amylase.

I further concur with your conclusion that the Ethyl 4 AA enzyme preparation that is manufactured using *B. licheniformis* BML612-Ethyl-4-CAP75 (GICC03266) by the process you described, in a manner consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food/feed grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid in grain and tuber starch processing, brewing, cereal beverage manufacture, as well as in potable alcohol and fuel ethanol with resulting co-products (corn gluten feed/meal, brewers' grains, distillers' grains) used as animal feed, where the enzyme is either not present in the final food/feed or present as inactive protein in insignificant quantities having no function or technical effect in the final food/feed.

It is my professional opinion that other qualified experts would concur in this conclusion.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject products by you or others.

Sincerely,

(b) (6)

Michael W. Pariza, Ph.D.
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Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

SUBMISSION END